

GLYCOSYLATION VARIANTS OF IDURONATE 2-SULFATASE

91 1. FIELD OF THE INVENTION

5 P The present invention relates to glycosylation variants of iduronate-2-sulfatase and to genetic sequences encoding same. The present invention also contemplates the use of these in the treatment and diagnosis of subjects suspected of, or suffering from, iduronate 2-sulfatase deficiency.

10 2. BACKGROUND TO INVENTION

15 P Iduronate 2-sulfatase (hereinafter abbreviated to "IDS"; EC 3.1.6.13) acts as an exosulfatase in lysosomes to hydrolyze the C2-sulfate ester bond from non-reducing-terminal iduronic acid residues in the glycosaminoglycans heparan sulfate and dermatan sulfate (1). IDS is one of a family of at least nine
20 sulfatases that hydrolyze sulfate esters in human cells. They are all lysosomal enzymes that act on sulfated monosaccharide residues in a variety of complex substrates with the exception of microsomal steroid sulfatase (or arylsulfatase C), which acts on sulfated 3 β -hydroxysteroids (1,2). Each sulfatase displays absolute substrate specificity, making the sulfatase family an attractive model
25 to investigate the molecular requirements for substrate binding and the catalysis of sulfate ester hydrolysis.

30 P A deficiency in the activity of IDS in humans leads to the lysosomal accumulation of heparan sulfate and dermatan sulfate fragments and their excretion in urine (1). This storage results in the clinical disorder Hunter syndrome (mucopolysaccharidosis type II, MPS-II) in which patients may present with variable phenotypes from severe mental retardation, skeletal deformities, and stiff joints to a relatively mild course (1). It has been postulated that this clinical heterogeneity reflects different mutations at the
35 IDS locus affecting enzyme expression, stability, or function. MPS-II is one of the most common mucopolysaccharidoses and is the only one that is X chromosome-linked (1).

P In accordance with the present invention, there is provided the nucleotide sequence for a full length cDNA clone for IDS from human endothelial cells. The present invention also provides the genomic clone for IDS. More particularly, following expression of the IDS nucleotide sequence in particular cell lines, a glycosylation variant of IDS has been isolated which possesses *inter alia* improved half-life and/or improved uptake properties when compared to the naturally glycosylated molecule.

1. SUMMARY OF THE INVENTION

P 10 One aspect of the present invention provides a recombinant human iduronate 2-sulfatase (IDS) or a fragment thereof retaining enzymatic activity wherein said recombinant IDS or fragment thereof is more highly glycosylated than the naturally occurring enzyme or equivalent fragment on the naturally occurring enzyme.

15

P Another aspect of the present invention contemplates a method for treating a patient suffering from iduronate 2-sulfatase (IDS) deficiency said method comprising administering to said patient an effective amount of a recombinant human IDS or a fragment thereof retaining enzymatic activity wherein said recombinant IDS or fragment thereof is more highly glycosylated than the naturally occurring enzyme or equivalent fragment on the naturally occurring enzyme.

P Yet another aspect of the present invention is directed to a pharmaceutical composition useful in the treatment of patients suffering from iduronate 2-sulfatase (IDS) deficiency said composition comprising the more highly glycosylated IDS or enzymatically active fragment thereof referred to above and one or more pharmaceutically acceptable carriers and/or diluents.

P 30 Still yet another aspect of the present invention provides an isolated genomic DNA fragment carrying in whole or in part the IDS gene or a mutant or derivative thereof. The isolation of the genomic clone will enable gene

3

therapy and genetic analysis of IDS deficiency diseases.

4.1 BRIEF DESCRIPTION OF THE FIGURES

- Figure 1 is a representation of compiled nucleotide sequence of the IDS cDNA clones and the deduced amino acid sequence of the encoded protein. Amino acid sequence is shown in the one-letter code above the nucleotide sequence. Nucleotide and amino acid numbers are depicted on the right margin. Possible sites for peptidase cleavage of the signal peptide are indicated with arrows. Underlined amino acids are colinear with amino-terminal sequences (14 kDa, Pro-Arg-Glu-Leu-Ile-Ala-Tyr-Ser-Xaa-Tyr-Pro-Arg-Xaa-Xaa-Ile-Pro, determined by direct sequence analysis). Potential N-glycosylation sites are starred. A potential polyadenylation signal is doubly underlined.
- Figure 2 is a photographic representation showing: (A) Southern blot analysis of MPS-II DNA for deletions and rearrangement of the IDS gene. λ c2S15 was used to probe a Southern blot of Pst I-digested DNA samples from a normal male and female (lanes 9 and 10, respectively) and from severely affected MPS-II patients (lanes 1-8). The sizes (kb) of DNA molecular mass standards are shown in the right margin. (B) Northern blot of RNA from human placenta. The size (kb) of each RNA species is shown in the right margin.
- Figure 3 is a representation showing alignment of amino acid sequences of human IDS, human glucosamine 6-sulfatase (19), human galactose 3-sulfatase or arylsulfatase A (14), human N-acetylgalactosamine 4-sulfatase or arylsulfatase B (15), human steroid sulfatase or arylsulfatase C (20, 21), and sea urchin arylsulfatase (22) shown in lines 2, 6, A, B C and U, respectively. Amino acids identical in all sulfatases are boxed. Amino acids identical in the arylsulfatase activities (lines A, B, C, and U) are starred on the bottom line. The ringed residues in lines 2, 6 and B indicate the first amino-terminal amino acid in polypeptides produced by internal proteolysis. Underlined sequences are unique to each particular sulfatase sequence and underlined and starred sequences are blocks of conserved residues.

Figure 4 is a schematic representation showing the construction of a chimeric IDS cDNA. The full length IDS cDNA clone, pB12Sc17, is shown with the unique NotI, StuI and HincII restriction enzyme sites marked. The narrow open bar indicates plasmid vector sequence, the solid bar coding sequence and the large open bar non-coding sequence. The oligonucleotide sequence inserted in place of the sequence removed by NotI/StuI digestion is shown below with the unique XbaI restriction enzyme site and the ATG (Met) initiation codon indicated.

Figure 5 is a photographic representation of SDS/PAGE of recombinant (r) IDS. rIDS (lane 1) and molecular mass standards (lane 2) were reduced with DTE and electrophoresed as detailed in Example 2 and then Silver stained. The sizes of the molecular mass standards are indicated on the right of the figure and the estimated mass of the rIDS on the left. All masses are in kDa.

Figure 6 is a photographic representation showing SDS/PAGE of rIDS after treatment with endoglycosidase F. rIDS was treated with endoglycosidase F, reduced, electrophoresed and stained with Gradipure Colloidal Gel Stain. Lane 1 contains untreated rIDS and lanes 2 and 3 rIDS treated with 1 and 5 units of endoglycosidase F, respectively. Lane 4 contains molecular mass standards with the sizes, in kDa, indicated to the right of the figure.

Figure 7 is a representation of the genomic nucleotide sequence for the IDS gene.

25

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes human IDS or an enzymatically active fragment thereof. More particularly, the present invention is directed to the expression of such a nucleic acid molecule in a host cell which results in the recombinant IDS (rIDS) being more highly glycosylated relative to the extent of glycosylation of

the naturally occurring molecule.

When comparing the extent of glycosylation, the reference molecule is either naturally occurring IDS purified, for example, from human liver or may be a recombinant molecule produced in a cell line with an extent of glycosylation similar to the naturally occurring molecule. The critical comparison is not the glycosylation pattern *per se* but the extent to which the molecule is glycosylated.

P 10 Preferably, the more highly glycosylated IDS of the present invention has a molecular weight at least 5 kDa greater than the naturally occurring molecule or its recombinant equivalent, more preferably at least 10 kDa greater, even more preferably at least 15 kDa greater and still even more preferably at least 20-30 kDa greater. Accordingly, the more highly glycosylated IDS has a
15 molecular weight of approximately 65-95 kD or more preferably from about 70 to about 90 kDa depending on the host cell employed. In a most preferred embodiment, the molecular weight is about 90 kDa when produced in CHO-K1 cells or about 70 kDa when produced in CHO-Lec 1 cells.

P 20 Conveniently, the cDNA encoding IDS or its fragment is modified by replacing the 5' non-coding sequence with a portion of rat pre-pro-insulin leader sequence and inserted into an appropriate expression vector. The modified cDNA is then subject to expression in cell lines capable of more highly glycosylating the resulting recombinant molecule. Although the preferred cell
25 lines described herein are CHO-KI cells and CHO-Lec1 cells, it would be routine for one skilled in the art to select other cell lines and screen the resulting recombinant IDS to ascertain the extent of glycosylation. All cell lines resulting in a more highly glycosylated IDS are encompassed by the present invention.

- The "nucleic acid molecule" of the present invention may be RNA or DNA (eg. cDNA), single or double stranded and linear or covalently closed. The nucleic acid molecule may also be genomic DNA corresponding to the entire gene or a substantial portion thereof or to fragments and derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence or may contain single or multiple nucleotide substitutions, deletions and/or additions including fragments thereof. All such variations in the nucleic acid molecule retain the ability to encode a more highly glycosylated IDS when expressed in the appropriate host or an enzymatically active fragment of IDS. The enzymatic activity of the resultant molecule is readily ascertained by, for example, using the radiolabelled disaccharide substrate IdoA2S-anM6S of Bielicki et al (3).
- The nucleic acid molecule of the present invention may constitute solely the nucleotide sequence encoding human IDS or like molecule or may be part of a larger nucleic acid molecule and extends to the genomic clone of IDS. The non-IDS encoding sequences in a larger nucleic acid molecule may include vector, promoter, terminator, enhancer, replication or signal sequences or non-coding regions of the genomic clone.
- In its most preferred embodiment, the cDNA encoding ^{SEQ ID NO:1}IDS_A is as set forth in Figure 1 or having at least 60%, preferably at least 70% and even more preferably at least 80-90% similarity thereto. The genomic sequence encoding ^{IDS is SEQ ID NO:4}IDS_{iso} preferably as set forth in Figure 7 or having similarity thereto as defined above for the cDNA clone.
- The present invention is particularly directed to recombinant IDS in more highly glycosylated form as hereinbefore described. The recombinant IDS may comprise an amino acid sequence corresponding to the naturally occurring amino acid sequence or may contain single or multiple amino acid substitutions, deletions and/or additions. The present invention also extends

to fragments of the IDS molecule but which retain IDS activity. Such fragments are referred to herein as being "enzymatically active". Accordingly, this aspect of the present invention contemplates a highly glycosylated IDS molecule or enzymatically active fragments or derivatives thereof. The IDS molecule of the present invention, therefore, comprises parts, derivatives and/or portions of the IDS enzyme having enzymatic activity and being more highly glycosylated relative to the naturally occurring enzyme or equivalent fragment or derivative.

10 Advantageously, the recombinant highly glycosylated IDS is a biologically pure preparation meaning that it has undergone some purification away for other proteins and/or non-proteinaceous material. The purity of the preparation may be represented as at least 40% of the enzyme, preferably at least 60%, more preferably at least 75%, even more preferably at least 85% and still more preferably at least 95% relative to non-IDS material as determined by weight, activity, amino acid homology or similarity, antibody reactivity or other convenient means.

20 Amino acid insertional derivatives of IDS of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with the following Table 1:

8

TABLE 1

Suitable residues for amino acid substitutions

5	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
	Ala	Ser
	Arg	Lys
	Asn	Gln; His
	Asp	Glu
10	Cys	Ser
	Gln	Asn
	Glu	Asp
	Gly	Pro
	His	Asn; Gln
15	Ile	Leu; Val
	Leu	Ile; Val
	Lys	Arg; Gln; Glu
	Met	Leu; Ile
	Phe	Met; Leu; Tyr
20	Ser	Thr
	Thr	Ser
	Trp	Tyr
	Tyr	Trp; Phe
	Val	Ile; Leu
25		

Where the enzyme is derivatised by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like.

- 5 Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

10

- ¶ The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield synthesis) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at

- 15 predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently elsewhere described such as Sambrook *et al*, 1989 *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

20

- ¶ The derivatives of the IDS of the present invention include single or multiple substitutions, deletions and/or additions of any component(s) naturally or artificially associated with the IDS enzyme such as carbohydrate, lipid and/or
- 25 other proteinaceous moieties. All such molecules are encompassed by the expressions "mutants", "derivatives", "fragments", "portions" and "like" molecules. These molecules are enzymatically active and retain their more highly glycosylated form relative to the naturally occurring enzyme or equivalent derivative when produced in suitable host cells.

30

1 The present invention also extends to recombinant IDS molecules when fused
to other proteinaceous molecules. The latter may include another enzyme,
reporter molecule, purification site or an amino acid sequence which facilitates
5 transport of the molecule out of a cell.

1 In a most preferred embodiment, the present invention has an amino acid or
corresponding IDS cDNA nucleotide sequence substantially as set forth in
Figure 1 or having at least 40% similarity, preferably at least 60% similarity
10 thereto or more preferably at least 80% or 85-90% similarity thereto.

1 The present invention further contemplates antibodies to the more highly
glycosylated IDS. The antibodies may be polyclonal or monoclonal, naturally
occurring or synthetic (including recombinant, fragment (eg Fab Fragment) or
15 fusion forms). Such antibodies will be useful in developing immunoassays for
IDS and in distinguishing between molecules having an altered extent of
glycosylation. Preferably, therefore, the antibody is capable of binding the
more highly glycosylated form of IDS but not the naturally glycosylated form of
the molecule.

20 It is within the scope of this invention to include any second antibodies
(monoclonal, polyclonal or fragments of antibodies) directed to the first
mentioned antibodies discussed above. Both the first and second antibodies
may be used in detection assays. Furthermore, the first antibody may be used
25 with a commercially available anti-immunoglobulin antibody. An antibody as
contemplated herein includes any antibody specific to the more highly
glycosylated form of IDS but not to the normally glycosylated enzyme.

30 Both polyclonal and monoclonal antibodies are obtainable by immunization
with the enzyme or protein and either type is utilizable for immunoassays.
The methods of obtaining both types of sera are well known in the art.
Polyclonal sera are less preferred but are relatively easily prepared by injection

of a suitable laboratory animal with an effective amount of IDS, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoabsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are
5 generally less favoured because of the potential heterogeneity of the product.

P The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal
10 antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in Compendium of Immunology Vol II, ed. by Schwartz, 1981; Kohler and Milstein, Nature 256:
15 495-499, 1975; European Journal of Immunology 6: 511-519, 1976). Antibodies capable of also binding to the non-highly glycosylated form of IDS can be readily removed, for example, by immuno-adsorbant techniques.

P The assay for the highly glycosylated IDS may be accomplished in a number of
20 ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4, 424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include
25 direct binding of a labelled antibody to a target.

P Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the
30 present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the samples containing an IDS to be tested is brought into contact with the bound molecule. After a suitable

- period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex; a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another
- 5 complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of haptens.
- 10 Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention, the sample is generally a biological sample comprising biological
- 15 fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.
- 19 In the typical forward sandwich assay, a first antibody having specificity for the highly glycosylated IDS, or antigenic parts thereof, is either covalently or
- 20 passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-
- 25 known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25 °C) to allow binding of any
- 30 subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the haptens. The second antibody is linked to a

reporter molecule which is used to indicate the binding of the second antibody to the hapten.

1 An alternative method involves immobilizing the target IDS molecules in the
5 biological sample and then exposing the immobilized target to specific
antibody which may or may not be labelled with a reporter molecule.
Depending on the amount of target and the strength of the reporter molecule
signal, a bound target may be detectable by direct labelling with the antibody.
Alternatively, a second labelled antibody, specific to the first antibody is
10 exposed to the target-first antibody complex to form a target-first antibody-
second antibody tertiary complex. The complex is detected by the signal
emitted by the reporter molecule.

15 By "reporter molecule" as used in the present specification, is meant a
molecule which, by its chemical nature, provides an analytically identifiable
signal which allows the detection of antigen-bound antibody. Detection may
be either qualitative or quantitative. The most commonly used reporter
molecules in this type of assay are either enzymes, fluorophores or
radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent
20 molecules.

25 In the case of an enzyme immunoassay, an enzyme is conjugated to the second
antibody, generally by means of glutaraldehyde or periodate. As will be
readily recognized, however, a wide variety of different conjugation techniques
exist, which are readily available to one skilled in the art. Commonly used
enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase
and alkaline phosphatase, amongst others. The substrates to be used with the
specific enzymes are generally chosen for the production, upon hydrolysis by
the corresponding enzyme, of a detectable color change. Examples of suitable
30 enzymes include alkaline phosphatase and peroxidase. It is also possible to
employ fluorogenic substrates, which yield a fluorescent product rather than
the chromogenic substrates noted above. In all cases, the enzyme-labelled

antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a
5 qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

10

Alternately, fluorescent compounds, such as fluorecein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to
15 excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the
20 fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

25

The present invention further contemplates a method of treating a patient suffering from IDS deficiency said method comprising administering to said patient an effective amount of a recombinant human IDS or a fragment thereof retaining enzyme activity wherein said recombinant IDS or fragment
30 thereof is more highly glycosylated than the naturally occurring enzyme or equivalent fragment of the naturally occurring enzyme.

The highly glycosylated rIDS has enhanced uptake properties and/or a longer *in vivo* half-life and, hence, is more efficacious than the naturally glycosylated molecule.

5 Such a highly glycosylated IDS is as herein described. Generally, this aspect of the present invention can be accomplished using a pharmaceutical composition.

10 Accordingly, another aspect of the present invention contemplates a pharmaceutical composition useful in treating patients suffering from a deficiency in IDS such as in Hunter Syndrome, said composition comprising a recombinant human IDS or a fragment thereof retaining enzyme activity wherein said recombinant IDS or fragment thereof is more highly glycosylated than the naturally occurring enzyme or equivalent fragment of the naturally
15 occurring enzyme, said composition further comprising one or more pharmaceutically acceptable carriers and/or diluents.

20 The formulation of pharmaceutical composition is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pennsylvania, USA.

25 The active ingredients of a pharmaceutical composition comprising the highly glycosylated IDS or fragments thereof are contemplated to exhibit excellent therapeutic activity, for example, in treating Hunter Syndrome when administered in amount which depends on the particular case. For example, from about 0.5 ug to about 20 mg per patient or per kilogram of body weight of the patient per day, week, or month may be administered. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be
30 proportionally reduced as indicated by the exigencies of the therapeutic situation. Depending on the patient or other conditions more preferred dosages comprise 10µg to 10mg, 20µg to 5mg or 100µg to 1mg per patient or

per kilogram of body weight of the patient per administration. The composition may be administered in any convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of administration, the active ingredient which comprises a highly glycosylated IDS or fragment thereof may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredients. For example, due to the low lipophilicity of IDS, these may potentially be destroyed in the gastrointestinal tract by enzymes capable of cleaving peptide bonds and in the stomach by acid hydrolysis. In order to administer the IDS molecules by other than parenteral administration, they may be coated by, or administered with, a material to prevent its inactivation. For example, the IDS molecules may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes.

P The active compounds may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

P The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy

syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

P Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the highly glycosylated recombinant IDS molecules are suitably protected as described above, the active compound may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For

oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound.

- 5 The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared
10 so that an oral dosage unit form contains an effective amount of recombinant IDS as hereinbefore described.

- 15 The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a
20 flavoring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and
25 flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

30

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

10

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μ g to about 2000 mg includes 1.0 μ g to 200mg, 10 μ g to 20mg and 100 μ g to 10mg. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

20

EXAMPLE 1

CLONING OF IDS GENE

1. MATERIALS AND METHODS

P 5 Materials.

Form A of IDS was purified from human liver as described (3). Restriction endonucleases, polynucleotide kinase, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and M13 sequencing kits were from Boehringer Mannheim. GeneScreen *Plus* nylon filters were from DuPont/NEN. [γ -³²P]ATP (500 Ci/mmol; 1 Ci = 37 GBq), [α -³²P]dCTP (3000 Ci/mmol), and Multiprime DNA labeling kit were from Amersham. Oligo(dT)-cellulose and Sephadex G-50 were from Pharmacia P-L Biochemicals. The X chromosome genomic library LA0XNL01 was from the American Tissue Culture Collection, and the λ gt10 random-primed human colon cDNA library (1.5×10^6 independent clones) and the λ gt11 human endothelial cDNA library (2.1×10^6 independent clones) were from Clontech.

P Polypeptide Isolation and Sequencing.

Approximately 20 μ g of form A liver IDS was subjected to a SDS/polyacrylamide gel electrophoresis and transferred to an Immobilon P membrane (Millipore) (4) with modifications of overnight pre-electrophoresis of the SDS/polyacrylamide gel and the addition of 200 μ l of 100 mM sodium thioglycollate to the cathode buffer chamber before electrophoresis. The 42-kDa and the 14-kDa polypeptides were excised and directly amino-terminal sequenced by Bresatec (Adelaide, Australia).

P Library Screening.

A 49-mer oligonucleotide sequence, ^{SED ID NO: 4} (3'-ACTAGTAGCACCTGCTGGACGCCGGGAGGGACCCGCTGATGCTGCTGCA-5') was designed from the amino-terminal amino acid sequence, ^{SED ID NO: 5} (using residues 8-24 of TSALNVLLIIVDDLRLPSLGDYDDVL) of the 42-kDa IDS polypeptide. T4 polynucleotide kinase and [γ -³²P]ATP were used to end-label

the 49-mer to a specific activity greater than 10^7 cpm/ μ g for screening of the X chromosome library. The bacterial host used was NM538 and 2×10^5 recombinants were screened at a density of 30,000 plaque-forming units per 15cm plate. Positive clones were plaque-purified, DNA was isolated from lysates, and the inserts were separated on 1% w/v agarose and then analysis by Southern blotting using the labeled 49-mer as a probe. A 49-mer positive 1.6-kilobase (kb) *Hind*III genomic DNA fragment was labeled with [α - 32 P]dCTP using a Multiprime DNA labelling kit and used to screen the human colon cDNA library. Approximately 5×10^5 recombinants were screened at a density of 55,000 plaque-forming units per 15cm plate using the bacterial host C600. A 300-base-pair (bp) *Hind*III-*Eco*RI fragment from the 3' end of a 1.5-kb colon cDNA clone (λ c2S15) was labelled and used to screen the human endothelial cDNA library. The bacterial host used was NM538 and 5×10^5 recombinants were screened at a density of 40,000 plaque-forming units per 15cm plate.

¶ Nucleotide Sequencing.

¶ Sonicated DNA fragments generated from the 1.5-kb cDNA insert were subcloned into M13mp19 for nucleotide sequence analysis by the dideoxynucleotide chain-termination method by using the Klenow fragment of DNA polymerase I at 45 °C (5). Some internal regions of the 1.5-kb cDNA were sequenced using primers labeled at their 5' ends with [λ - 32 P]ATP with single-stranded DNA templates generated by asymmetric polymerase chain reactions. The remaining coding sequence and the 5' and 3' untranslated regions present on the 2.3 kb endothelial cDNA were sequenced using specific primers on M13 subclones.

¶ Southern Blot Analysis of MPS-II Patients.

¶ DNA from MPS-II patients and normal control cultured fibroblasts was prepared and digested with *Pst* I (6) and separated by agarose gel electrophoreses and transferred to GeneScreenPlus nylon membrane. The cDNA fragment λ c2S15 was radiolabeled using the Multiprime DNA labeling

kit and purified by gel filtration on a 1-ml Sephadex G-50 column. The nylon filter was prehybridized, hybridized, and washed according to the manufacturer's instructions.

P 5 RNA Isolation and Northern Blot Analysis.

P Total RNA was isolated from placental tissue by using a single-step guanidinium thiocyanate method (7). Poly(A)⁺ RNA was obtained by oligo(dT)-cellulose chromatography and characterized by Northern Blot analysis carried out after electrophoresis in a 0.8% w/v agarose/2.2 M

10 formaldehyde gel and transfer to GeneScreenPlus nylon membrane.

Prehybridization, hybridization, and washing were performed according to the manufacturer's instructions. Radiolabeled λ c2S15, prepared and purified as described above, was used in all hybridization experiments.

P 15 Sequence Analysis.

P The nucleotide sequence was screened against the GenBank nucleotide sequence data base (Release 62.0, December 1989) and the encoded protein sequence was screened against the National Biomedical Research Foundation protein data base (Release 23.0, December 1989). General sequence analysis
20 and the multiple protein sequence alignment were performed using programs from Reisner and Bucholtz (8) and Lipman *et al.* (9), respectively.

CL
2. RESULTS

P 25 IDS from human liver can be purified to two major forms (A and B) which have different pI values and contain both 42 kDa and 14 kDa polypeptides (3). The 42 kDa and 14 kDa polypeptides in form A were subjected to direct amino-terminal amino acid sequencing and a region of low codon redundancy in the 42 kDa amino-terminal sequence was used to design a single 49-mer
30 oligonucleotide sequence incorporating choices based on human codon usage (10). The 49-mer detected 14 clones when used to screen an X chromosome enriched genomic library. Two overlapping clones were analysed in more

detail and found to contain the same 1.6 kb 49-mer positive *Hind*III fragment. This fragment was shown to give a positive signal when used to probe DNA from a human-mouse cell hybrid that contained the tip of the long arm of the X chromosome (Xq26-ter) consistent with the localisation of the IDS gene to this small portion of the human X chromosome (1).

The 1.6 kd *Hind*III genomic DNA fragment was then used to screen a human colon cDNA library. Eighteen clones were detected and their inserts were sized. The clone with the longest insert (λ 2S15) was fully sequenced and found to contain an initiating methionine and a continuous open reading frame that included a sequence that was colinear with the 42 kDa and the 14 kDa amino-terminal amino acid sequences. However, the reading frame did not extend to include a stop codon or any 3' untranslated region. A 300 bp *Hind*III-*Eco*RI restriction fragment from the 3' end of the λ c2S15 was then used to screen a cDNA library constructed from human endothelial cells. Twenty seven clones were isolated; 5 of which were also positive to the amino-terminal-specific 49-mer. Of the five, the clone that contained the longest insert (2.3 kb; λ c2S23) was sequenced in combination with λ c2S15,

Figure 1 shows the nucleotide sequence of the 2297 bp insert from λ 2S23, which encodes the entire amino acid sequence of IDS. Except for a few differences, the deduced amino acid sequence was colinear with the determined amino-terminal amino acid sequence of the 42 kDa and 14 kDa polypeptides. The amino acid discrepancies (residues 35, 53, 55 and 57) between the direct and predicted amino acid sequence data are believed to reflect amino acid sequencing errors resulting from the low signal obtained toward the end of the amino acid sequencing run. The detection of gene deletions and rearrangements in DNA from a group of severely affected MPS-II patients when hybridised with λ c2S15 established that these cDNA clones encoded IDS (Figure 2A). Of the 23 MPS-II patients analysed, 7 had structural alterations including deletions of the entire λ c2S15 coding region. These 7 patients also revealed similar Southern patterns indicative of structural

alterations of the IDS gene when their DNA was digested with *Hind*III, *Stu* I and *Taq* I and probed with λ c2S15. Sixteen patients had identical patterns to normal controls, suggesting the presence of small deletions or point mutations responsible for the MPS-II biochemical and clinical phenotype. The two

- 5 patients, in which the entire IDS gene had been removed (Figure 2A) had the most severe clinical phenotype of the large group of MPS-II patient studied, raising the possibility that these patients may also have deletions of contiguous genes to IDS.

- 10 The sequence of λ c2S23 shown in Figure 1, ^(SER ID NO: 2) contains an open reading frame from the initiation codon at position 125 to the termination codon (TGA) at position 1775. This 1650 bp sequence encodes a polypeptide of 550 amino acids as shown.

- 15 ^{SER ID NO: 1} The sequence flanking the ATG codon at bp 125_A is in agreement with the consensus sequence for initiator codons (11). The first 25 amino acids at the amino terminus of the deduced protein have features characteristic of a signal sequence (12). Two putative sites for cleavage between the signal sequence and mature protein are indicated by arrows (Figure 1). It would appear that
- 20 eight amino acids are removed from the IDS precursor immediately after the most favored signal peptidase cleavage site (12) between residues 25 and 26. The 14 kDa polypeptide amino-terminal amino acid sequence was identified at amino acid residue 456, giving a total of 95 amino acids to the carboxyl terminus. The full sequence contains eight possible N-glycosylation sites (Asn-
- 25 Xaa-Ser/Thr, Figure 1). The molecular weight of the deduced polypeptide for the 14 kDa component was calculated as 11,093. The 14 kDa polypeptide does not contain cysteine residues, which is compatible with the finding that the 42 kDa and the 14 kDa polypeptide are not linked by disulfide bonds (3). The number of potential N-glycosylation sites used in the 42 kDa polypeptide is not
- 30 known. The first N-glycosylation site (residue 31) is not contained within IDS form A since this asparagine residue is removed during amino-terminal processing. The molecular weight of the deduced peptide for the 42 kDa

25

component was calculated as 47,404, suggesting that the value determined by SDS/polyacrylamide gel electrophoresis (3) may be in error or that additional amino acids are lost during internal proteolytic cleavage of the IDS precursor. These results suggest that post-translational proteolytic processing of IDS is restricted to cleavage of a signal peptide, removal of the amino-terminal 8 amino acids, and internal cleavage to produce the observed 42 and 14 kDa polypeptides in human liver, kidney, lung and placenta (3). This is a commonly observed polypeptide maturation process for lysosomal enzymes that are generally synthesised as larger precursors and then converted to their mature forms by a limited number of proteolytic steps shortly before or after their transfer into lysosomes (13).

¶ Northern blot analysis of placental poly (A)⁺ RNA with λ c2S15 revealed three major RNA species (5.7, 5.4 and 2.1 kb) and one minor species (1.4 kb) (Figure 2B). It is likely that IDS, like other lysosomal enzymes [e.g., arylsulfatase A, B, and C (14-16)], has mRNA species that differ in length at their 3' ends due to differential polyadenylation. Arylsulfatase C has three major RNA transcripts that result from the use of different polyadenylation sites (2.7, 5.2 and 7.0 kb) the longest of which has a 3' untranslated region of >4 kb (16). Differential polyadenylation can account for the three major species but it cannot explain the 1.4 kb minor species, which is too small to encode the full IDS protein. It is possible that the 1.4 kb species represents a degradation product or a cross-reacting species, although it is also possible that it results from a process of differential splicing to produce another protein product, as has been observed for the human lysosomal enzymes, for example, β -glucuronidase (17) and β -galactosidase (18). The 520 bp of 3' untranslated region in λ c2S23 contains a potential polyadenylation signal (AATAAA) at position 2041 that may direct the position of polyadenylation for the observed 2.1 kb mRNA species. If this is the case, the 124 bp of 5' untranslated region in λ c2S23 is sufficient to account for most, if not all, of the 5' untranslated region expected for the 2.1 kb mRNA species [allowing for 50-100 residues of poly(A) tail].

26

Figure 3 shows an alignment of IDS amino acid sequence with sequence of other human-derived sulfatases and a sea urchin arylsulfatase. This analysis reveals many areas of identical and conserved amino acid matches within the arylsulfatase group (galactose 3-sulfatase, N-acetylgalactosamine 4-sulfatase and steroid sulfatase) and the two nonarylsulfatase sequences (unpublished data), IDS and glucosamine 6-sulfatase. Sea urchin arylsulfatase is also aligned and has sequence homology with the other five human sulfatases. A multiple sequence alignment of the amino acid sequence of these six sulfatases has the highest level of homology in the amino-terminal third of each sulfatase (Figure 3). The human arylsulfatase group has conserve blocks of up to six identical amino acid residues, for example, Cys-Thr-Pro-Ser-Arg and Gly-Lys-Trp-His-Leu-Gly (Figure 3). On the other hand, only part of these sequences are conserved in the two nonarylsulfatases, IDS and glucosamine 6-sulfatase. These sequences may represent regions of the arylsulfatases that enable the relatively nonspecific hydrolysis of arylsulfates. All five human sulfatases have significant sequence homology with the amino acid sequence of sea urchin arylsulfatase (Figure 3). By taking account of conservative amino acid substitutions (23), there are even larger areas of homology within these six sulfatases. This high level of sequence conservation further supports the suggestion that these five human sulfatases are evolutionarily related to a common ancestral gene (14, 15, 19).

There are several regions in Figure 3 where peptide inserts appear to be unique to a particular sulfatase. For instance, the microsomal membrane-bound steroid sulfatase contains two membrane-spanning regions (Figure 3) (21). IDS also contains an amino acid sequence insert in the same region as the second membrane-spanning region of steroid sulfatase (Figure 3). A second peptide insert in IDS is present just before the amino terminal sequence of the 14 kDa polypeptide. The role that these two peptide inserts may have in IDS function is unknown. Interestingly, the sites (ringed in Figure 3) for internal proteolysis of both glucosamine 6-sulfatase (19) and N-acetylgalactosamine 4-sulfatase also occur near the sequence inserts.

27

(and see ID NO: 2)

The genomic sequence for IDS was isolated and is set forth in Figure 7.

CL

EXAMPLE 2

PRODUCTION OF HIGHLY GLYCOSYLATED FORMS OF IDS

5

1. MATERIALS AND METHODS

9 All enzymes for DNA manipulations, DNAase, dithiothreitol, kanamycin and streptomycin were purchased from Boehringer Mannheim (Dulwich, SA, Australia). DNA oligonucleotides were synthesised using an Applied biosystems 391 DNA Synthesiser. $\text{Na}_2^{35}\text{SO}_4$ (516 mCi/mmol) was purchased from New England Nuclear (Dupont, North Ryde, NSW, Australia). PBE94 chromatofocusing medium, polybuffer 74 and high and low molecular-mass standard kits for SDS-PAGE and gel chromatography were obtained from Pharmacia (North Ryde, NSW, Australia). TSK G3000SW Ultrapac was purchased from LKB (Bromma, Sweden). Blue A matrix agarose gel and ultrafiltration stirred cell model 8200 and Diaflo ultrafiltration membrane YM10 was obtained from Amicon (Danvers, MA, USA). Dialysis membrane with a 10-12 kDa cut off was obtained from Union Carbide Corp. (Chicago, IL, USA). Endoglycosidase F was purchased from Nenzymes (DuPont Co., Wilmington, DE USA). Dulbecco's modified phosphate-buffered saline (PBS) was purchased from Commonwealth Serum Laboratories (Melbourne, Vic, Australia). Nonidet P40, mannose-6-phosphate and BSA were purchased from Sigma (St. Louis, MO, USA). Basal medium Eagle's (BME), penicillin and glutamine were obtained from Flow Laboratories (Sydney, NSW, Australia) and fetal calf serum (FCS), Ham's F12 nutrient mixture, CHO-SFM medium and G418 (Geneticin) were from Gibco (Glen Waverley, Vic., Australia).

10 DNA Manipulation and Recombinant Plasmids

11 All DNA preparation, modification and cloning procedures were done using standard techniques (26). The IDS cDNA clone pB12Sc17 contains bp 107 (NotI restriction enzyme site) to bp 1870 (BstXI restriction enzyme site) of the IDS cDNA of Example 1, cloned between the NotI and EcoRV restriction

(see ID NO: 2)

28

enzyme sites of pBlueScript (Stratagene, La Jolla, CA, USA). The expression vector pRSVN.08 was derived from pRSVN.07 (27) by the introduction of an EcoRV site into the polylinker such that the order of restriction sites is 5' *HindIII*, *XbaI*, *BamHI*, *EcoRV*, *EcoRI*, *NodI* 3'.

5

P Culture and Electroporation of CHO-K1 cells

P CHO-K1 cells were cultured and electroporated as previously described (17) unless otherwise stated. Lec 1 cells are available from the New Jersey Cell Line Collection, New Jersey, USA. Under ATCC CRL 1735 and are described
10 in Stanley *et al Somat Cell Genet.* vol 3 (1977) pp 391-405.

P Culture of fibroblasts

P Human diploid fibroblasts were established from skin biopsies submitted to this hospital for diagnosis (28). Cell lines were maintained according to
15 established procedures in BME, 10% v/v FCS and antibiotics unless otherwise stated. The two MPS II skin fibroblast cell lines used in this study (SF-635 and SF-1779) both have low residual IDS activity.

P Determination of IDS expression

20 P Media samples, or cell lysates prepared by six cycles of freeze/thaw in 0.5 M-NaCl/20 mM-Tris/HCl, pH 7.0, were clarified by microcentrifugation (12,000 x g, 4°C, 5 min) and were either assayed directly or after dilution in assay buffer. Where possible cell lysates were dialysed in 5 mM-sodium acetate, pH 4.0, before assaying as this results in higher measured enzyme activity. IDS
25 was assayed using the radiolabelled disaccharide substrate IdoA2S-anM6S (3). Protein estimations were according to the method of Lowry *et al* (29).

P β -Hexosamidase

P The fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside was used to measure β -hexosaminidase activity (31).
30

Correction of MPS II fibroblasts

For these experiments IDS was obtained from CHOEFI2S-9 cells cultured in CHO-SFM medium supplemented with 10 mM-NH₄Cl and antibiotics. The medium was concentrated 10-fold by ultrafiltration and was shown to contain rIDS with activity of 2.75×10^6 pmol/min per ml (133 μ g of I2S/ml).

Fibroblasts from a normal individual (SF-3409) and from two MPS II patients (SF-635 and SF-1779) were grown to confluency in 25 cm² flasks and radiolabelled with Na₂³⁵SO₄ as previously described (27). The labelled cells

were then exposed to 5×10^4 pmol/min per ml of rIDS for 72 hours. After harvesting the cells by trypsin treatment and washing by centrifugation/resuspension in PBS, the cell pellet was resuspended in 100 μ l of 20 mM-Tris/HCl, pH 7.0/0.5 M-NaCl, and the cell lysates prepared as described above. The cell extracts were analysed for IDS activity, total

protein, β -hexosaminidase activity and radioactivity.

Endocytosis of rIDS

Cells from SF-1779 were plated in 20 wells (3.83 cm²) and allowed to reach confluency. Wells 1 to 4 were untreated controls. To each of wells 5 to 12 and 13 to 20 was added 1.0 ml of medium containing rIDS at 5×10^4 pmol/min per ml and 5×10^3 pmol/min per ml respectively. In addition the medium in wells 9 to 12 and 17 to 20 was made 5 mM mannose-6-phosphate. The cells were then incubated for 6 hours after which time they were rinsed with medium and fresh medium added. The cells were incubated overnight and then harvested, washed and lysed as described above. The cell lysates were dialysed against 5 mM-sodium acetate, pH 4.0, for 16 h at 4 °C and then analysed for IDS activity and total protein.

Subcellular fractionation

Cells from SF-635 were grown to confluency in 75 cm² flasks and then exposed to medium supplemented with 5 x 10⁴ pmol/min per ml rIDS. The cells were incubated for 72h then harvested and fractionated on Percoll density gradients as described in Anson *et al* (27). The resulting gradient was collected in 1.0 ml fractions by bottom puncture and the fractions analysed for IDS and β -hexosaminidase activity.

10 Large-scale production of rIDS

CHOEFI2S-9 cells were inoculated into two 2-layer cell factories (NUNC, 1200 cm²) in Ham's F12, 10% v/v FCS and antibiotics. Cells were grown to confluency, the medium removed and the cells were then rinsed 3-times with PBS and re-fed with 200 ml of Ham's F12 without FCS but supplemented with antibiotics and 10 mM-NH₄Cl. After 4 days in culture, the medium was collected and replaced with Ham's F12, 10% v/v FCS and PSK but without NH₄Cl for 3 days. This cycle was repeated several times. The conditioned serum free Ham's F12 medium supplemented with NH₄Cl was collected, clarified by filtration (0.2 μ m filter; Millipore) and stored at 4 °C.

The rIDS was purified from the collected medium by a 3-step column procedure. The medium was dialysed overnight at 4 °C against 30 mM-Tris/HCl, pH 7.0/10% v/v glycerol/0.1 mM-DTE/3 mM-NaN₃ (buffer A) and was applied to a PBE94 column (8 cm x 1.5 cm) equilibrated in buffer A (flow-rate 1.0 ml/min) and then washed with 100 ml of buffer A. Bound proteins were diluted with polybuffer 74 that had been diluted 1:18 with water, the pH adjusted to 4.0 with HCl and the solution made 10% v/v in glycerol, 0.1 mM-DTE and 3 mM-NaN₃. The column was further eluted with 100 ml 15 mM-dithiothreitol/3 mM-NaN₃ (buffer B). The rIDS eluted in buffer B was applied at a flow-rate of 1.0 ml/min to a Blue A agarose column (6 cm x 0.7 cm) also equilibrated in buffer B. The rIDS activity from this step was applied in 1.0 ml volumes to an LKB Ultrachrom GTi f.p.l.c. system with a

TSK G3000SW Ultrapac column (30 cm x 0.8 cm) equilibrated and eluted in buffer B at a flow-rate of 0.5 ml/min and pressure of 150 kPa. Fractions containing rIDS activity were pooled and analysed under denaturing and non-denaturing condition on SDS-PAGE (10% w/v acrylamide) to estimate
5 apparent subunit size. Gels were stained with either Gradipure Colloidal Electrophoresis Gel Stain (Gradipure, Pyrmont, NSW) or silver stained according to the method of Merril *et al* (32). Native molecular mass was determined using the f.p.l.c. system as described elsewhere (3) Kinetic (Km, Vmax, pH optima) and inhibition data were obtained as previously described
10 (3).

Endoglycosidase F treatment of IDS

To two identical 60 µl samples, each containing 2.5 µg of rIDS, was added an equal volume of buffer containing 100 mM-sodium phosphate, pH 6.1/50 mM-
15 EDTA/1% v/v Nonidet P40/0.1% v/v SDS/1% v/v 2-mercaptoethanol. After boiling both samples for 5 min, to one was added 1 unit and to the other 5 units of endoglycosidase. Both samples were incubated for 17 h at 37 °C. A control sample was untreated but stored in similar buffer conditions at 4 °C. Bromophenol blue was added to each sample before analysis on SDS-PAGE.
20 Molecular-mass standards were applied to SDS-PAGE in the same buffer as the enzyme samples.

2. RESULTS

25 Construction of IDS expression vectors

An initial expression construct containing an IDS cDNA from pB12Sc17 cloned into pRSVN.08 expressed I2S at very low levels when introduced into CHO-K1 cells. A chimeric I2S cDNA was then made by replacing the 5' non-coding sequence of the I2S cDNA with 45bp of the rat preproinsulin leader sequence
30 (Figure 4) as an analogous chimeric N-acetylgalactosamine-4-sulphatase cDNA construct resulted in the expression of high levels of enzyme activity in the same system (27). Briefly, the sequence shown in Figure 4 was synthesised as

two complementary oligonucleotides which were then kinased and annealed. The resulting double stranded fragment was then cloned between the dephosphorylated NotI and StuI sites of pB12Sc17. The resulting construct was designated pB12SNC.1. The IDS cDNA insert was then excised from

5 pB12SNC.1 with XbaI and HincII and cloned into XbaI/EcoRV restricted and dephosphorylated pRSVN.08 resulting in the construct pRSVN.2SNC1. In order to further increase expression of rIDS the chimeric rIDS cDNA was placed under the transcriptional control of the human elongation factor-1 α (EF-1 α) gene promoter. This was done by excising the RSV-LTR from

10 pRSVN.2SNC1 by SalI/XbaI digestion and inserting the HindIII/XbaI fragment from pEF-BOS (32), after making the HindIII and SalI ends blunt by filling in with the Klenow fragment of DNA polymerase I. This construct was designated pEFN.2SNC1. Both pRSVN.2SNC1 and pEFN.2SNC1 were electroporated into CHO-K1 cells and G418 resistant clonal cell lines isolated.

15 Individual clones were assayed for secretion of IDS activity into the culture medium. Replacement of RSV-LTR promoter with EF-1 α promoter resulted in a 2-fold enhancement of IDS expression. A clonal cell line, CHOEFI2S-9, was selected on the basis of maximum expression of IDS activity. This clone secreted IDS such that after 5 days of culture approximately 11 mg of IDS

20 accumulated per litre of medium.

P Large-scale production of rIDS

f Conditioned serum-free Ham's F12 medium containing NH₄Cl was collected as described above. Enzyme was collected in this manner to facilitate purification

25 by minimising total protein in medium. As prolonged exposure to this medium resulted in loss of cell viability the cells were cycled in Ham's F12 with 10% v/v FCS to allow recovery. A total of 1 litre of serum free medium, containing approximately 11 mg of rIDS was collected in this manner.

30 **f** The rIDS bound very tightly to PBE94 medium and not not eluted in significant amount during polybuffer elution (less than 10% of the total enzyme recovered from this column was eluted with polybuffer, pool A). The

33

majority of rIDS (pool B) had a pI of <4.0 and required NaCl for elution. Enzyme was eluted in buffer B in concentrated form (essentially in one 10 ml fraction). This permitted direct application to Blue A agarose. Although the rIDS did not bind to this matrix it was a necessary step to remove some minor

5 contaminating proteins which were observed after f.p.l.c. when the enzyme from the chromatofocusing step was applied directly to f.p.l.c. Recovery of activity from Blue A agarose was 80%. The final step in the purification (f.p.l.c.) resulted in overall recovery of greater than 15% activity. The estimated native molecular mass on f.p.l.c. was 90 kDa. A single diffuse

10 protein band of 80-92 kDa was observed when a sample from the f.p.l.c. step was subjected to SDS-PAGE (Figure 5). This diffuse band was observed on SDS-PAGE run under reducing or non-reducing conditions indicative of a single subunit species with no disulphide bonding. Correlation of the protein species observed as a diffuse band on SDS-PAGE with IDS activity was

15 demonstrated by PAGE run under non-reducing conditions, according to the method of Laemmli (33), but with the modification that SDS was omitted from all buffers. Identical amounts of enzyme were applied to 2 lanes of the gel. One lane was stained for protein and as with SDS-PAGE a single diffuse band was observed. The other was cut into 2 mm slices and each slice was

20 incubated in 4-times the volume of assay mix at 37 °C overnight. When corrected for swelling which occurred during the staining procedure, the position of the diffuse band corresponded to that of IDS activity in the lane that was sliced and assayed.

25 The molecular size of IDS (after cleavage of the signal peptide) estimated from cDNA sequence data indicated a maximum of 58 kDa with 7 potential glycosylation sites (see Example 1). The mature or processed forms of IDS had various molecular sizes depending on the column matrix used. The native molecular size varied from 42 kDa to 65 kDa while, on a denaturing SDS-

30 PAGE, two polypeptide bands of 43 kDa and 14.4 kDa were consistently observed. The recombinant form of IDS had a markedly larger molecular size (80-90 kDa; Figure 5) than predicted. The diffuse nature of the Coomassie-

34

stained band on SDS-PAGE implied that the protein was highly and variably glycosylated. To test the hypothesis that the difference in the observed Mr and the expected estimated value was due to carbohydrate, rIDS was treated with endoglycosidase F as outlined above. Treatment with 1 unit of

5 endoglycosidase F resulted in a decrease in Mr (70 kDa - 80 kDa). However, the enzyme still migrated as a diffuse band on SDS-PAGE (Figure 6, lane 1). Lane 2, which shows the result of treatment with 5-times the concentration of endoglycosidase F, demonstrates the presence of a tightly staining 60 kDa protein band with a diffuse band above it (62 kDa to 68 kDa). Other bands
10 are due to endoglycosidase F.

P These data suggest that the 60 kDa band is the end product of the deglycosylation of rIDS by endoglycosidase F and that the diffuse bands in both lanes are the result of incomplete digestion. Endoglycosidase F cleaves
15 the glycosidic bond between GlcNAc residues of the chitobiose core in the N-linked carbohydrate chains resulting in one GlcNAc residue remaining linked to asparagine. This would account for approximately 1540 kDa due to carbohydrate if all 7 of the glycosylation sites were utilised and may therefore account for the molecular size of IDS after endoglycosidase F treatment as
20 being 60 kDa rather than 58 kDa.

P Kinetics of rIDS

P Although both the liver and rIDS show a similar Km towards the disaccharide substrate (IdoA2S-anM6S) in the standard assay (50mM sodium acetate pH 4.5
25 and 500 µg/ml BSA) they have a substantially different Vmax. This suggests that the recombinant form of the enzyme may be less efficient in turning over the substrate than the mature form. Alternatively, this may reflect a difference between enzyme produced in CHO cells and in liver. Both the (CHO) recombinant and (liver) mature form of the enzyme have similar pH optima
30 and specific activities (Table 2).

35

Inhibition studies showed that the rIDS was similar to the liver enzyme with regard to inhibition by sulphate, phosphate and copper ions. The rIDS appears to be less sensitive to salt inhibition than liver enzyme (Table 3).

Demonstration of correction of MPS II fibroblasts

Fibroblasts from patients with MPS II store undegraded HS and DS fragments. This storage is reflected in the accumulation of labelled material when the cells are metabolically labelled with $\text{Na}_2^{35}\text{SO}_4$. Supplementing culture medium with rIDS at 5×10^4 pmol/min per ml resulted in clearance of this stored product to levels comparable to those seen in control fibroblasts (Table 4) and to levels of IDS activity 40- to 80-fold above normal in SF1779 and SF635 respectively. The activity of a second lysosomal enzyme, β -hexosaminidase, was not affected by endocytosis of IDS (Table 4).

To test whether endocytosis of the rIDS occurs via the mannose-6-phosphate receptor MPS II cells (SF-1779) were cultured in medium supplemented with rIDS at 5×10^4 and 5×10^3 pmol/min per ml in the presence or absence of 5 mM mannose-6-phosphate. Inhibition of the uptake of IDS activity by mannose-6-phosphate at both doses of enzyme confirmed that uptake is mediated via the mannose-6-phosphate receptor.

Localisation of endocytosed rIDS

Endocytosed rIDS was instrumental in correcting the lysosomal storage in MPS II skin fibroblasts, as demonstrated by the loss of accumulated S^{35} -labelled material. Confirmation of the subcellular localisation of the endocytosed enzyme was demonstrated by fractionating the post-nuclear supernatant of corrected and control MPSII skin fibroblasts on Percoll gradients as described above. Analysis of these gradients showed that in the corrected MPS II cells, IDS activity fractionated with the lysosomal enzyme β -hexosaminidase in the dense fraction of the gradient. Control MPS II fibroblasts contained no detectable levels of IDS activity and a similar β -hexosaminidase activity profile.

36

37

TABLE 2
Comparison of the Catalytic Properties of Recombinant and Liver IDS

	Km (μ M)	Vmax (μ mol/min per mg)	Specific Activity (μ mol/min per mg)	pH Optimum
Liver IDS	4.0	80	11.9	4.5
rIDS	3.0	3.35	20.8	4.5

TABLE 3
Comparison of the Effect of Various Inhibitors on Recombinant and Liver IDS

	NaCl (mM)	Na ₂ SO ₄ (μ M)	Na ₂ HPO ₄ (μ M)	Cu Acetate (mM)
Liver IDS	40	50	30	15
rIDS	160	115	35	8

Values shown are for 50% inhibition of IDS activity. For details, see Materials and Methods section.

TABLE 4

Correction of the MPS II Defect by Recombinant IDS

	IDS (pmol/min per mg)	β -Hexosaminidase (nmol/min per mg)	³⁵ S-cpm/mg Cell Protein
SF-3409	13.5 \pm 2.2 (n=3)	83.0 \pm 7.8 (n=3)	3138 \pm 491 (n=3)
SF-1779	n.d. (n=3)	150 \pm 10 (n=3)	196927 \pm 21247 (n=3)
SF-1779 + rIDS	562 \pm 99 (n=3)	118 \pm 11 (n=3)	5136 \pm 502 (n=3)
SF-635	1.6 \pm 1.5 (n=3)	269 \pm 29 (n=3)	233080 \pm 66010 (n=3)
SF-635 + rIDS	1140 \pm 50 (n=3)	257 \pm 14 (n=3)	9018 \pm 1988 (n=3)

n = number of experimental repeats;

n.d. = none detected

Normal and MPS II fibroblasts were labelled with Na₂³⁵SO₄ and exposed to 5 x 10⁴ pmol/min per ml of rIDS as described in Materials and Methods. Undialysed cell lysates were analysed for IDS activity, total protein, β -hexosaminidase activity and radioactivity.

CL REFERENCES:

1. Neufeld, E.F. & Muenzer, J. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D. (McGraw-Hill, New York), pp. 1565-1587.
2. Shapiro, L.J. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D. (McGraw-Hill, New York), pp. 1945-1964.
3. Bielicki, J., Freeman, C., Clements, P.R. & Hopwood, J.J. (1990) *Biochem, J.*, 271: 75-86.
4. Matsudaira, P., (1987) *J. Biol. Chem.* 262: 10035-10038.
5. Sanger, R., Nicklen, S., & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.
6. Nelson, P.V., Carey, W.F., Morris, C.P., & Pollard, A.C. (1989) *Med. J. Aust.* 151: 126-131.
7. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* 162: 156-159.
8. Reisner, A.H., & Bucholtz, C. (1987) *Nature (London)* 314: 310.
9. Lipman, D.J., Altschul, S.F. & Kecicioglu, J.D. (1989) *Proc. Natl. Acad. Sci. USA* 86: 4412-4415.
10. Masuyama, T., Gojobori, T., Aota, S. & Ikemura, T. (1986) *Nucleic Acids Res.* 14: r151-t197.

- P 11. Kozak, M. (1987) *Nucleic Acids Res.* 15: 8125-8131.
12. von Heijne, G. (1986) *Nucleic Acids Res.* 14: 4683-4690.
- L 13. Hasilik, A. & von Figura, K. (1984) in *Lysosomes in Biology and Pathology* eds. Dingle, J.T. & Sly, W.S. (Elsevier, Amsterdam) Vol. 7, pp. 3-16.
- P 14. Stein, C., Gieselmann, V., Kreysing, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H.E., O'Brien, J.S. & von Figura, K. (1990) *J. Biol. Chem.* 265: 3374-3381.
- P 15. Peters, C., Schmidt, B., Römmskirch, W., Rupp, K., Zuhlsdorf, M., Vingron, M., Meyer, H.E., Pohlmann, R. & von Figura, K. (1990) *J. Biol. Chem.* 265: 3374-3381.
- P 16. Yen, P.H., Marsh, B., Allen, E., Tsai, S.P., Ellison, J., Connolly, L., Neiswanger, K. & Shapiro, L.J. (1988) *Cell* 55: 1123-1135.
- P 17. Oshima, A., Kyle, J.W., Miller, R.D., Hoffman, J.W., Powell, P., Grubb, J.H., Sly, W.S., Tropak, M., Guise, S. & Gravel, R.A. (1987) *Proc. Natl. Acad. Sci. USA* 84: 685-689.
- P 18. Morreau, H., Galjart, M.J., Gillemands, N., Willemsen, R., van Horst, T. J. & d'Azzo, A. (1989) *J. Biol. Chem.* 264: 20655-20663.
- P 19. Robertson, D.A., Freeman, C., Nelson, P.V., Morris, C.P. & Hopwood, J.J. (1988) *Biochem. Biophys. Res. Commun.* 157: 218-224.

20. Yen, P.H., Allen, E., Marsh, B., Mohandas, T., Wang, N., Taggart, R.T. & Shaapiro, L.J. (1987) *Cell* 49: 443-454.
21. Stein, C., Hille, A., Seidel, J., Rijnbout, S., Waheed, A., Schmidt, B., Geuze, H & von Figura, K. (1989) *J. Biol. Chem.* 254: 13865-13872.
22. Sasaki, H., Yamada, K., Akasaka, K., Kawasaki, H., Suzuki, K., Saito, A., Sato, M. & Shimada, H. (1988) *Eur. J. Biochem.* 177: 9-13.
23. Dayhoff, M.O., Schwartz, R.M. & Orcatt, B.C. (1978) in *Atlas of Protein Sequence and Structure*, ed. Dayhoff, M.O. (Natl. Biomed. Res. Found., Washington, DC) Vol. 5, Suppl. 3, pp 345-352.
24. Lee, G.D. & van Etten, R.L. (1975) *Arch. Biochem. Biophys.* 171: 424-434.
25. James, G.T. (1979) *Arch. Biochem. Biophys.* 197: 57-62.
26. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) *Current Protocols in Molecular Biology*, Wiley Interscience, New York.
27. Anson, D.S., Taylor, J.A., Bielicki, J., Harper, G.S., Peters, C., Gibson, G.J. and Hopwood, J.J. (1992) *Biochem. J.* 284: 789-794.
28. Hopwood, J.J., Muller, V., Harrison, J.R., Carey, W.F., Elliott, H., Robertson, E.F. and Pollard, A.C. (1982) *Med. J. Aust.* 1: 257-260.
29. Lowry, O.H., Rosebrough, N.H. Farr, A.L. and Randell, R.J. (1951) *J. Biol. Chem.* 193: 265-275.

42

- 43